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Highly Sensitive Spectrofluorimetric Assay of Thiamine in Food

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A highly sensitive and simple spectrofluorimetric method for the determination of thiamine based on the fluorescence quenching effect of thiamine on the hemoglobin-catalyzed reaction of H_2O_2 with L-tyrosine was developed. The concentration of thiamine is linear with the fluorescence quenching (ΔF) of system under the optimal experimental conditions. The calibration graph is linear in the range 2.37×10^{-8}-9.49×10^{-5} mol L^{-1} with the detection limit of 5.53×10^{-9} mol L^{-1}. This method can be used for the determination of thiamine in food with satisfactory results.

Key Words: Thiamine, Spectrofluorimetry, Hemoglobin.

INTRODUCTION

Thiamine is an important water-soluble vitamin taking part in glycometabolism in the body. It has been used for the prevention and treatment of beriberi, neuralgia, *etc*., in medical doses or thiamine-enriched food or drinks. Therefore, the determination of thiamine is one of the important contents in food and clinical analysis. Several methods have been already reported for the quantitative determination of thiamine in food and clinical analysis, including UV-visible spectrophotometry[1-4], spectrofluorimetry[5,6], chemiluminescence[7,8], high-performance liquid chromatography[9] and electrochemical method[10,11]. However, some methods lack sensitivity and selectivity, others are tedious and time-consuming.

Enzyme-catalyzed analytical kinetic methods have been extensively used for substrate, enzyme, inhibitor and activator analysis in several areas of analytical chemistry such as in clinical, pharmaceutical, agricultural, industrial applications and process monitoring[12]. Horseradish peroxidase (HRP; EC 1.11.1.7) is one of the most important oxidases in biology. Having the function of active molecular oxygen, horseradish peroxidase can enhance the oxidation of H_2O_2 directly into H_2O. However, natural enzymes do have shortcomings in some aspects; *e.g.*, it is expensive and unstable in solution and has strict requirements for the experimental conditions and storage environment in order to retain its catalytic activity. Hemoglobin (Hb), a necessary vehicle for oxygen carriage in body, has the natural quaternary structure as enzymes. In a recent paper, hemoglobin was used based on its similar catalytic function as horseradish peroxidase[13].

In this paper, a new spectrofluorimetric method based on the fluorescence quenching effect of thiamine on the hemoglobin-catalyzed reaction of H_2O_2 and L-tyrosine is proposed. The experimental conditions for the system were optimized and thiamine was detected by the decrease fluorescence. The method has been applied to the determination of thiamine in food with satisfactory results.

EXPERIMENTAL

Hemoglobin (bovine erythrocytes) solution was prepared by dissolving certain amount of hemoglobin (Shanghai Institute of Biochemistry, Shanghai, China) in distilled water and stored below 4 ºC. L-Tyrosine (Beijing Chemical Plant, Beijing, China) stock solution was prepared by dissolving 0.0906 g of L-tyrosine in 500 mL of water, which was 10^{-3} mol L^{-1} in L-tyrosine. H_2O_2 solution was prepared by appropriately diluting 0.01 mL of 30 % H_2O_2 (standardized by titration with $KMnO_4$) to 100 mL. It was stored in a brown bottle in a refrigerator. Thiamine (Shanghai Aobo Institute of Biochemistry, Shanghai, China) solution was prepared to the concentration of 3.00×10^{-3} mol L^{-1}. Working solution was diluted appropriately before use with distilled water daily. *Tris*-HCl buffer solutions of different pH was used throughout the present study. Doubly distilled water was used throughout. All other chemicals were of analytical-reagent grade.

The spectrofluorimetric detection was carried out on a FP-750 spectrofluorimeter (JASCO). The temperature was controlled by using a TB-85 thermostat bath (Shimadzu) and the pH values were measured with a pHS-3C precision pH meter (Shanghai, China).

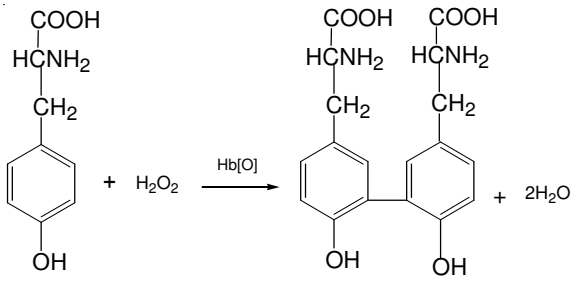
Each color comparison tube was filled with 2.00 mL of pH 7.87 *tris*-HCl buffer solutions, 2.50 mL of 1.0×10^{-3} mol L^{-1} L-tyrosine, a proper amount of thiamine solutions, 1.00 mL of 1.0×10^{-5} mol L^{-1} Hb and 1.20 mL of 1.0×10^{-3} mol L^{-1} H_2O_2 and then diluted with water to 10 mL. After being equilibrated in a thermostated water bath (25 ± 0.2 ºC) for 20 min, the difference of the relative intensity (ΔF) between the blank (F_0) and the sample (F) was measured at the selected maximum excitation wavelength of 316.0 nm and maximum emission wavelength of 406.0 nm. Then the value of $\Delta F = F_0 - F$ was calculated.

An amount equivalent to 10 g of milk powder was weighed accurately and added 70 mL of 0.1 mol L^{-1} HCl solution into a 250 mL conical flask. The mixture was shaked well and hydrolyzed in 0.5 h in a pressure cooker of 1 Mpa. After cooling, the solution was adjusted to 5-6 of pH by 6 M NaOH and dissolved in a 100 mL calibrated flask. The solution was filtered and collected to reserve by brushing off first filtration.

After washing and mashing, an amount equivalent to 13 g of pork liver was weighed accurately into a 250 mL conical flask. Following processing procedure is according to the procedure of milk powder.

RESULTS AND DISCUSSION

Hemoglobin-catalyzed reaction is shown below:



L-tyrosine Fluorescence dimer

In this redox reaction between H_2O_2 and L-tyrosine, different amounts of thiamine had fluorescence quenching effects on Hb-catalyzed reaction, which leaded to the decrease of the relative fluorescence intensity of the whole system. The fluorescence spectra of the fluorescence dimer with and without thiamine are shown in Fig. 1. The difference of them is remarkable. In addition, there is a good linearity between the amounts of thiamine and ΔF, on which this new method was based.

The variable and ranges studied and their optimum values, are summarized in Table-1.

It is noted that thiamine has less effect in assay involving higher concentrations of hemoglobin. The ΔF increased with increase in hemoglobin concentration at first, but decreased above 1.00×10^{-6} mol L^{-1}. It might be due to the loss of substrate inhibition, which occurs at high hemoglobin concentration, which could be due to the inability of thiamine to promote conformational changes when hemoglobin is at high concentration. So 1.00×10^{-6} mol L^{-1} of hemoglobin was selected for further work.

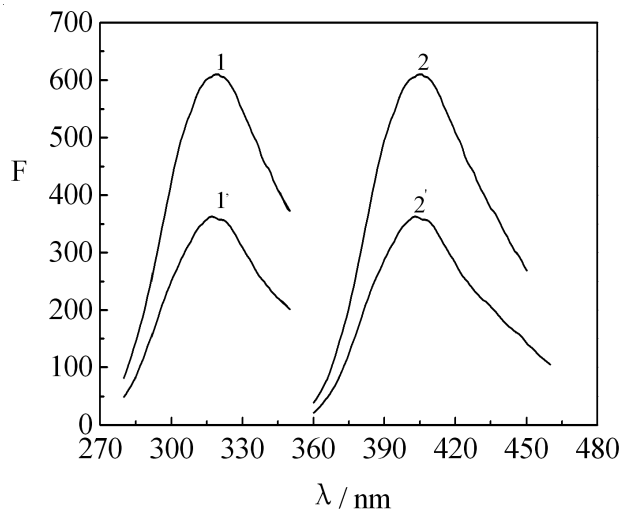


Fig. 1. Fluorescence spectra of the system

TABLE-1
OPTIMIZATION STUDY FOR THIAMINE DETERMINATION BY FLUORESCENCE QUENCHING ON Hb-CATALYZED REACTION

Variable	Range studied	Recommended value
pH	7.20-8.23	7.87
Hb (mol L^{-1})	$0.50\text{-}2.00 \times 10^{-6}$	1.00×10^{-6}
H_2O_2 (mol L^{-1})	$0.50\text{-}2.00 \times 10^{-4}$	1.20×10^{-4}
L-tyrosine (mol L^{-1})	$1.00\text{-}4.00 \times 10^{-5}$	2.50×10^{-5}
Temperature (ºC)	10-50	25
Time (min)	1-35	20

The effect of H_2O_2 concentration on inhibition was studied. The ΔF increased with the increase in H_2O_2 up to 1.20×10^{-4} mol L^{-1}, above which it had little effect. Thus 1.20×10^{-4} mol L^{-1} H_2O_2 was selected for further study. The ΔF was greatest at pH 7.87. Considering the fluorescence intensity getting too weak at very low L-tyrosine concentration, 2.50×10^{-4} mol L^{-1} L-tyrosine was chosen for further study.

The effect of temperature on the system was investigated in a range from room temperature to 50 ºC. The time needed to reach equilibrium, 20 min, was prolonged with the decreasing temperature. Due to the decomposition of H_2O_2 at high temperature, temperature was kept at 25 ºC and the measurements were carried out after 20 min.

From the results obtained under the recommended conditions (Table-1), it was found that the ΔF of thiamine on the Hb-catalyzed reaction was linear in the range 2.37×10^{-8}-9.49×10^{-5} mol L^{-1}. The linear response can be fitted to an equation as follows:

$$\Delta F = (54.6226 \pm 5.0577) + (26.5115 \pm 1.1494)\left[\frac{c}{10^{-5}}\right]$$

(r = 0.9935, n = 9)

c = concentration of thiamine in mol L^{-1}. r and n are the linear correlation coefficient and the number of experiments, respectively. The detection limit, calculated according to the $3S_b/k$ criterion (in which "k" is the slope over the range of linear used and "S_b" is the standard deviation (n = 11) of the signal from the blank), was found to be 5.53×10^{-9} mol L^{-1}. The relative standard deviation for 11 replicate determination of 5.93×10^{-5} mol L^{-1} thiamine was 2.38 %. The existing methods

for the determination of thiamine are summarized in Table-2. It can be seen that the proposed method has higher sensitivity.

TABLE-2
COMPARISON OF EXISTING METHOD FOR THE DETERMINATION OF THIAMINE WITH PROPOSED METHOD*

Methods of determination	Detection limit (mol L^{-1})	Linear range (mol L^{-1})	References
FL (EI)	5.53×10^{-9}	2.37×10^{-8}-9.49×10^{-5}	Proposed method in this paper
UV-Vis	–	0-1.80×10^{-6}	2
UV-Vis	2.97×10^{-6}	7.42×10^{-6}-1.48×10^{-4}	4
FL	–	5.93×10^{-6}-2.97×10^{-4}	5
CL	1.48×10^{-8}	1.48×10^{-7}-2.97×10^{-5}	7
FI-CL	2.97×10^{-8}	1.48×10^{-7}-2.37×10^{-5}	8
ECL	2.97×10^{-7}	5.93×10^{-7}-8.97×10^{-5}	10

*UV-Vis: ultraviolet spectrophotometry; EI: enzymatic inhibition; FL: fluorimetry; CL: chemiluminescence; FI: flow injection; ECL: electrochemical analytical method.

Several common amino acids, reducing compounds and vitamins were investigated for their interference for the determination of 5.93×10^{-5} mol L^{-1} thiamine. When the permitted relative deviation is larger than ± 5.0 %, the examined species may cause a significant alteration in the results. The results are shown in Table-3. It can be seen that the proposed method has good selectivity.

TABLE-3
EFFECT OF VARIOUS SPECIES ON Hb ACTIVITY

Species	Tolerance ratio
K^+, Na^+, Cl^-, Ca^{2+}, F^-, PO_4^{3-}, NO_3^-, NH_4^+, Mg^{2+}, starch, dextrin, glucose, fructose	1000
OAc^-, glycine, serine	500
Mn^{2+}, Cu^{2+}, cysteine	100
Fe^{3+}	10

The current method was applied to determine thiamine in food by using the procedure described in the experimental section. The filtered solutions of thiamine were diluted to different concentrations with double distilled water, so the final concentration was in the working range for further sample analysis. In order to evaluate the validity of the proposed method, precolumn derivation-HPLC method was also used for the determinations by closely following a procedure described in the literature[14]. The results obtained by the two different methods were statistically compared in Table-4. It can be seen that no significant differences were found between them. It is indicated that the method is able to determine thiamine in food.

TABLE-4
DETERMINATION AND RECOVERY TEST OF THIAMINE IN FOOD

Samples	Current method* (10^{-5} mol L^{-1})	Precolumn derivation-HPLC method (GB/T 1997) (10^{-5} mol L^{-1})	RSD (%)	t**
Pork liver	0.60 ± 0.05	0.62 ± 0.04	3.26	2.65
Milk power	2.53 ± 0.02	2.51 ± 0.03	1.93	2.19

*Mean ± standard deviation of five determination. **Theoretical value = 2.78, n = 5, with 95 % confidence limits.

Conclusion

A new spectrofluorimetric method for trace amount of thiamine determination was developed based on fluorescence quenching effect of thiamine on Hb-catalyzed reaction. The current method is simple, sensitive and the detection limit is 5.53×10^{-9} mol L^{-1}. The current method can be used for the determination of thiamine in food with satisfactory results.

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